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Polyethylene Glycol-Induced Precipitation of Interferon Alpha-2a Followed By Vacuum Drying: Development of a Novel Process for Obtaining a Dry, Stable Powder

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ABSTRACT

Feasibility studies were performed on the development of a novel process based on polyethylene glycol (PEG)-induced precipitation of proteins followed by vacuum drying in the presence of sugars to obtain dry protein powders. Apparent solubility of interferon alpha-2a (IFNα2a) was determined in the presence of various PEGs and the effect of solution pH, ionic strength, and temperature was investigated. IFNα2a precipitate was dried at a shelf temperature of 25°C at 100 mTorr either as it is or in the presence of mannitol and/or trehalose. The dried IFNα2a formulations were subjected to accelerated stability studies at 40°C (3 months), and the stability was compared with that of a similar lyophilized formulation. The results indicated that more than 90% of the protein could be precipitated using 10% wt/vol PEG 1450 at pH 6.5 at a solution ionic strength of 71 mM. Vacuum drying of the precipitate only resulted in the formation of insoluble aggregates of IFNα2a; however, this was prevented by the addition of either mannitol or trehalose. The addition of excess mannitol resulted in low residual moisture content and better handling of the final dried product. Accelerated storage stability did not show any aggregation and showed less than 5% formation of oxidized IFNα2a in the dried formulation containing IFNα2a:trehalose:mannitol in a 1:10:100 wt/wt ratio upon storage at 40°C for 3 months. The stability of this vacuum dried formulation was comparable with that of a similar lyophilized formulation.

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INTRODUCTION

Because of the enhanced physical and chemical instability of proteins in aqueous solutions,[1-5] it is a common practice to formulate proteins as dry powders to achieve sufficient stability for the desired shelf life of the product. Traditionally, lyophilization has been the process of choice for the formulation of proteins as dry powders.[6,7] Recently, there has been an increased level of interest in developing drying technologies other than lyophilization. The need has been generated to overcome some of the key issues associated with the process of lyophilization.[8-13] These include long processing times (typically 3-5 days), expensive set up and maintenance of the lyophilization plants, and most of all, the instabilities incurred upon proteins because of the inherent steps of lyophilization.

The alternative technologies to lyophilization that have been reported in literature include spray-drying,[14-15] spray-freeze drying,[16] bulk crystallization,[17] supercritical fluid technology,[18-19] vacuum drying,[20-21] and foam drying.[22] Although these processes have been shown to overcome some of the problems associated with lyophilization, several limitations still exist in each of these processes. For example, the presence of large air/water interface has been associated with protein aggregation in spray drying.[15] Similarly, the use of supercritical fluids involves organic solvents that may not be favorable for maintaining protein structure,[18] and simple vacuum drying may not result in an acceptable powder form of the product.[21] It is reasonable to state that while new processes continue to emerge to formulate proteins as dry powders, there are still problems associated with these processes that need to be addressed. From the protein formulation point of view, an "ideal" process would be

the one that is economical, does not create protein stability problems during the processing of formulation, and produces stable protein powders for any given protein for the desired shelf life of the product.

The principle objective for any drying process is the removal of water, which is achieved either by sublimation or by evaporative drying at high temperatures and/or at low vacuum pressures. One of the techniques that has been used to phase-separate proteins out of aqueous solutions as suspensions is precipitation of proteins using salts (ammonium sulfate), organic solvents (ethanol and acetone), or nonionic polymers (polyethylene glycol [PEG] or dextran).[23-29] The phenomenon of phase separation of proteins provides a simple means of concentration of proteins from aqueous solutions, as most of the water can be removed by filtration or centrifugation. The above-mentioned precipitants can be used for protein precipitation as long as the final objective is purification, and there is a step involved to remove the precipitant by redissolving or dialysis or by some other suitable technique. However, before a suitable precipitant is chosen to induce precipitation with the intent of drying protein precipitate for stable protein formulation for therapeutic use, there are certain criteria that must be met. The desired protein precipitant must include the following properties: (1) it should not cause denaturation of the protein during and following precipitation; (2) it should not be added in large amounts to achieve maximum precipitation, since there will not be any step to remove the precipitant from the formulation; (3) the protein should preserve its native biological activity upon reconstitution of the precipitate; and (4) it must be approved for parenteral use.

From the list of the various protein precipitants, it was observed that PEG could be used as a protein precipitating agent for formulation purposes, since it satisfies most of the criteria (higher molecular weight PEG can affect tertiary structure of proteins as observed in the present studies) outlined above.[23,30-34] In fact, PEG has been reported to carry out crystallization of proteins for therapeutic use.[17] PEG has several advantages over other precipitants, including least denaturation of proteins at ambient temperatures (does not affect the native structure of the protein in solution as compared with organic solvents), negligible temperature control required in the range of 4°C to 30°C, relatively small amount (5%-10% wt/vol) of precipitant required compared with ammonium sulfate (4 M) or organic solvents (40%-50% vol/vol), and low residual PEG concentration in the precipitate since most of the PEG is retained in the supernatant.[23,32]

The theory of protein precipitation by PEGs has been discussed in detail elsewhere.[35,36] In general, the precipitation of proteins by PEGs is explained on the basis of the volume exclusion effects according to which proteins are sterically excluded from the regions of solvent that are occupied by PEG linear chains. Proteins are thus concentrated and finally precipitated when the solubility is exceeded. In thermodynamic terms, the steric exclusion leads to an increase in the chemical potential of the protein until it exceeds that of the "pure solid state," leading to precipitation of the protein. This happens mainly because of a large unfavorable free energy of interaction between PEG and proteins leading to preferential hydration of protein attributed to the steric exclusion effects. The preferential hydration helps maintain the native structure of proteins in the presence of PEG.

In the present study, a novel process has been proposed for the formulation of proteins as dry powders, which is based on the precipitation of proteins by nonionic polymers such as PEGs followed by vacuum drying of the wet precipitate in the presence of stabilizers such as sugars. The overall objective of this study was to develop a process that can be conveniently used for any given protein and at the same time is economical, less time consuming, and preserves the protein structure and conformation during the shelf life of the product.

For the feasibility studies, recombinant human interferon alpha-2a (IFNα2a), currently marketed in the solution form by Hoffman-La Roche Inc (Nutley, NJ), was used as the model protein. IFNα2a, one of the many subtypes of interferons, contains 165 amino acids with 4 cysteines and 2 disulfide linkages and has a molecular weight of 19.225 kDa. The 3-D solution structure of IFNα2a has been determined by nuclear magnetic resonance (NMR) spectroscopy at pH 3.5, and it was revealed that IFNα2a contains mainly alpha helices (6 alpha helices, 65% alpha helical) in its secondary structure and the rest is random coil and loops.[37] The pI of IFNα-2a lies in the pH range of 6.0 to 7.0. IFNα2a is known to undergo aggregation and oxidation upon storage in solution conditions.[38]

This study determined that PEG-induced precipitation of proteins followed by vacuum drying can be used as a suitable process to formulate proteins as dry powders. Results are presented in detail on the optimization of precipitation of IFNα2a by PEGs, on the development of the process for the drying of the precipitate, and on the stability studies of various formulations that were prepared by PEG-induced precipitation of IFN-a2a followed by vacuum drying. Finally, results from the accelerated storage stability studies on PEG-precipitated and dried IFNα2a are compared with that of a similar lyophi-

lized IFNα2a formulation in order to compare the long-term stability of dry protein powders produced by the 2 methods.

MATERIALS AND METHODS

Materials

All buffer reagents and chemicals used in the present studies were of highest purity grade available from commercial sources and were used without further purification. PEGs 400 to 8000 were obtained from Dow Chemical Co (Danbury, CT). D-mannitol was obtained as fine crystalline powder United States Pharmacopeia grade from Cerestar USA Inc (Hammond, IN). Trehalose dihydrate was obtained from Sigma-Aldrich (St Louis, MO). IFNα2a was donated generously by Hoffman-La Roche and was supplied as 1.6 mg/mL solution in 25-mM acetate buffer, pH 5.0, containing 120-mM NaCl (total ionic strength = 142 mM). The protein was stored in several aliquots at -80°C, and each vial was thawed at 4°C before use.

Solubility Studies

The solubility of IFNα2a in the presence of various molecular weight PEGs was obtained as a function of PEG concentration and solution pH, ionic strength, and temperature. The effect of PEG molecular weight and concentration was studied at 25°C at pH 6.5, which is the midpoint of the isoelectric pH range of IFNα2a. The pH of IFNα2a was adjusted to 6.5 using 1.0-M NaOH solution. The PEG solutions were buffered at pH 6.5 using 25-mM phosphate buffer with added NaCl to maintain the total ionic strength at 142 mM. Briefly, IFNα2a was precipitated using different molecular weight PEGs (PEG 1450, 3650, 4600, and 8000) by adding aliquots of buffered PEG stock solutions to 0.5 mL of IFNα2a solution (final IFNα2a concentration was 0.5 mg/mL) to vary PEG concentration from 1% to 25% wt/vol. The final volume was made up to 1 mL. The effect of ionic strength was studied for PEG 1450 and 4600 at 142-mM (original ionic strength) and 71-mM (obtained by dilution) solution ionic strength. The ionic strength of all buffer solutions was adjusted using NaCl. The effect of pH on solubility of IFNα2a was studied in the pH range of 5.0 to 9.0 in presence of 10% wt/vol PEG 1450. Since the final solutions had a mixed acetate-phosphate buffer, the solutions exhibited good buffer capacity in this pH range except for pHs 8.5 and 9.0. To investigate the effect of temperature, the solubility of IFNα2a was determined in the presence of PEG 1450 (1%-25% wt/vol) at 5°C, 25°C, and 37°C.

The final solutions were mixed well and equilibrated for 2 hours and then centrifuged at 5000 rpm for 30 minutes to separate the protein precipitate. Protein solubility was obtained by measuring absorbance of the supernatant at 280 nm and calculating the concentration based on an $E^{1\%}$ of 9.3. Since the precipitates obtained were amorphous in nature (based on the literature that rapid achievement of supersaturation results in the formation of amorphous precipitates),[39,40] the solubility obtained by using the above mentioned procedure presented apparent solubility of IFNα2a rather than the true solubility under given solution conditions. This "apparent solubility" simply represented the concentration of the protein in the supernatant under defined solution conditions.

Structural Characterization of the IFNα2a Precipitate

The precipitate obtained after addition of PEG was collected by centrifugation followed by removal of the supernatant. Based on volumetric calculations ~90% of initial PEG was removed during this step. Protein secondary structure in the precipitate was compared with the protein secondary structure in solution using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). The precipitate was reconstituted in acetate buffer, pH 5.0, and the secondary structure and the tertiary structure of the reconstituted IFNα2a was compared with the structure of native IFNα2a obtained from Hoffman-La Roche using far UV and near UV circular dichroism (CD) spectroscopy.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

ATR-FTIR spectra of native IFNα2a and PEG-precipitated IFNα2a were recorded in the region of 4000 to 400 cm^{-1} at 25°C on a Jasco-410 FTIR (Jasco, Inc, Portland, OR) equipped with an ATR accessory and a nitrogen-cooled deuterated tryglycine sulfate (dTGS) detector at a resolution of 2 cm^{-1}. The spectrometer was continuously purged with dry nitrogen. The protein solution or wet precipitate (~20 μL obtained after centrifugation) was spread on the germanium ATR plate (50 × 20 × 2 mm and an aperture angle of 45°) by slowly evaporating the sample using dry nitrogen. A total of 128 scans were averaged and corrected for the background using a clean ATR plate. The original scans were smoothed using an 11 cm^{-1} smoothing function and the second derivatives of these scans were obtained in the 1750 to 1550 cm^{-1} region (amide I). The second derivative spectra were baseline subtracted and finally area-normalized to unit area for relative comparisons.[41,42]

CD Spectroscopy

CD measurements were performed using a Jasco-710 Spectropolarimeter. The far UV CD studies were performed in a 0.05-cm path length cell using a protein concentration of 0.25 mg/mL and a scan speed of 20 nm/min from 185 to 260 nm. The near UV CD studies were performed in a 1-cm path length cell using a protein concentration of 0.5 mg/mL and a scan speed of 50 nm/min from 240 to 310 nm. A total of 5 spectra were accumulated and averaged.

Vacuum Drying Studies

The precipitates that exhibited similar secondary and tertiary structures upon reconstitution to that of the native IFNα2a solution were subjected to vacuum drying. Briefly, protein precipitates were collected after centrifugation and removal of the supernatant and subjected to vacuum drying at a controlled shelf temperature of 25°C and at a chamber pressure of 100 mTorr in an FTS Durastop lyophilizer (FTS/Kinetics Thermal Systems, Stone Ridge, NY) for a period of 48 hours. Initially, drying studies were performed on wet precipitate only. In another set of studies, mannitol or trehalose was added in various ratios to the wet protein precipitate ranging from 1:1 to 1:100 wt/wt IFNα2a:sugar. The sugars were added in dry form to the wet precipitate in a vial or a microcentrifuge tube and mixed well with the precipitate using a spatula. This mixing process allowed some of the sugar to dissolve in the wet precipitate (when added up to 1:10 wt/wt protein:sugar ratio, most of the sugar seemed to be dissolved), and the excess sugar remained as it is with the wet precipitate spread all over the sugar particles.

The drying process was characterized for moisture content using Karl Fischer titrimetry during and after the drying, protein secondary structure in the dried precipitates using transmission FTIR spectroscopy, presence of soluble or insoluble aggregates upon reconstitution of the dried protein precipitates using UV spectroscopy and size exclusion chromatography, and secondary and tertiary structure of the protein in the reconstituted samples using CD spectroscopy (methodology described above).

Moisture Analysis

Moisture content was determined using an Orion AF7LC Coulometric Karl Fischer Titrimeter (Orion Research Inc, Boston, MA). Samples were analyzed by dissolving or dispersing precipitates in methanol and by using dry methanol as the blank.

Analysis of Protein Secondary Structure

The secondary structure of IFNα2a in vacuum-dried samples was evaluated by obtaining area-normalized second derivative FTIR spectra using a Nicolet Magna 560 FTIR spectrometer (Nicolet Inc, Madison, WI) equipped with a dTGS detector. The spectra of dried protein powders were collected in the transmission mode using KBr pellets prepared by mixing samples containing equivalent of 0.25 to 1 mg protein in 150 mg of dried KBr under dry nitrogen environment. A total of 100 scans were accumulated in the 4000 to 400 cm^{-1} region at 4 cm^{-1} instrument resolution and apodized using Happ-Genzel function. The original scans were smoothed using an 11-point Savitzky-Golay window, and the second derivatives of these scans were obtained in the 1750 to 1550 cm^{-1} region (amide I). The second derivative spectra were baseline corrected and finally area-normalized to unit area for relative comparisons.[41,42]

Estimation of Insoluble and Soluble Aggregates

The amount of insoluble aggregates that may have formed during drying was determined after reconstitution of the vacuum-dried samples followed by filtration through a 0.45-μm filter. The concentration of the protein in the filtrate was determined by measuring A_{280} and was compared with the protein concentration in the reconstituted and filtered wet precipitate. The difference provided information about the insoluble aggregates in the dried samples. Precipitation by itself did not induce formation of any soluble or insoluble aggregates in the reconstituted samples as reconstitution of the wet precipitate before drying did not show presence of soluble or insoluble aggregates (data not shown).

The amount of soluble aggregates was determined by analyzing the reconstituted and filtered samples using size exclusion high-performance liquid chromatography (HPLC) attached online to a UV detector set at 280 nm and a 90° light scattering detector by Precision Detectors (Bellingham, MA). A Waters YMC-Pack Diol-60 column, DL0S06S05-3008WT (300 mm × 8.0 mm inner diameter, 5 μm particle size), which is a diol end-capped silica-based column (Waters Corp, Milford, MA), was used for IFNα2a. The mobile phase used was 25-mM acetate buffer, pH 5.0, with 125-mM NaCl and the flow rate used was 1.0 mL/min.

Stability Studies

Accelerated stability studies on PEG-precipitated and vacuum-dried IFNα2a were performed at 40°C for 3 months. All samples were stored in desiccators contain-

ing anhydrous calcium sulfate (Fisher Scientific, Pittsburgh, PA) to maintain 0% relative humidity, and the desiccators were placed in 40°C storage oven. The control samples were kept at -20°C. Stability studies were performed on different formulations of vacuum-dried precipitate containing IFNα2a only; IFNα2a and mannitol (1:100 wt/wt); IFNα2a and Trehalose (1:100 wt/wt); and IFNα2a, mannitol, and trehalose (1:100:10 wt/wt). For comparison, a lyophilized formulation containing 1:10:100 wt/wt IFNα2a:trehalose:mannitol was prepared in an FTS Durastop lyophilizer and subjected to stability studies. The lyophilization cycle was as follows: samples were cooled to 0°C for 15 minutes and then frozen at -45°C for 2 hours. The shelf temperature was increased to -15°C at 2°C/min and the chamber pressure was decreased to 100 mTorr for 48 hours. The shelf temperature was then increased to 25°C at 2°C/min and the vacuum was retained at 100 mTorr. The secondary drying was performed for 12 hours. The stability of samples at various time points was assessed for the formation of insoluble aggregates by UV spectroscopy (as described in the "Estimation of Soluble and Insoluble Aggregates" section above), for soluble aggregates by size exclusion chromatography (SEC) (methodology described above) and for oxidized species of IFNα2a using reversed-phase (RP)-HPLC.

The RP-HPLC studies for identification of oxidized IFNα2a were performed using a 300 Å, 150 × 2.1 mm inner diameter Vydac C_{18} column, 218TP5215 (Grace Vydac, Hesperia, CA), attached to a UV detector with wavelength set at 220 nm. Separation of the oxidized IFNα2a was achieved by using a gradient elution of 95% mobile phase A (0.2% trifluoroacetic acid (TFA) in water) and 5% mobile phase B (0.2% TFA in acetonitrile) at 0 minutes to 5% mobile phase A and 95% mobile phase B over 95 minutes. The oxidized IFNα2a was identified by comparing the chromatograms of the samples to that obtained by the oxidation of IFNα2a solution in acetate buffer, pH 5.0, using 100-mM H_2O_2 for 5 minutes.

RESULTS AND DISCUSSION

Solubility Studies

The solubility of IFNα2a can be affected by PEG molecular weight and concentration, and solution ionic strength, pH, and temperature. Hence, the purpose of the solubility studies of IFNα2a in the presence of PEG was to optimize experimental solution conditions that can provide maximum yield of the precipitated protein without compromising native protein structure upon reconstitution.

Figure 1 shows the effect of PEG average molecular weight (PEG 1450, 3350, and 4600) and concentration (2%-25% wt/vol) and the effect of solution ionic strength (70 mM and 142 mM) on the solubility of IFNα2a at pH 6.5 and 25°C. At a fixed solution ionic strength of 142 mM, the solubility of IFNα2a decreased nonlinearly with an increase in PEG concentration for different molecular weights of PEG. The decrease in the solubility was observed to be almost 10-fold with the lowest solubility observed around 0.04 mg/mL under different solution conditions studied. With an increase in the PEG molecular weight, the IFNα2a solubility curve as a function of PEG concentration shifts to the left indicating that a lower concentration of a higher molecular weight PEG is required to achieve similar solubility of IFNα2a. For example, the solubility of IFNα2a decreased from 0.8 mg/mL for a 5% wt/vol PEG 1450 solution to 0.4 mg/mL for a 5% wt/vol PEG 4600 solution. The solution ionic strength had a tremendous effect on the solubility of IFNα2a in the presence of PEG. A decrease in the ionic strength from 142 mM to 71 mM decreased the solubility of IFNα2a significantly, thus shifting the whole solubility-concentration curve to the left. This decrease in solubility is attributed to the "salting-in" effect of salts on protein solubility and has been well recognized for several proteins.[43] The decrease in the solubility of IFNα2a upon lowering of the solution ionic strength was observed for both PEGs (ie, 1450 and 3350).

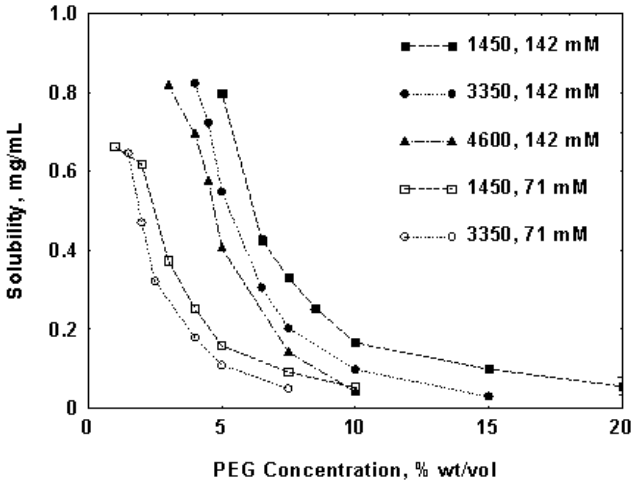

Figure 1. Effect of PEG molecular weight, concentration, and solution ionic strength on the apparent solubility of IFNα2a at 25°C, pH 6.5. The data points represent mean of the duplicate studies and the lines are guide to the eyes. The difference between the 2 values in duplicate studies was less than 5% of the mean.

The impact of solution ionic strength on protein solubility in the presence of PEG is considered important for the present studies, since the solubility of IFNα2a can be easily manipulated by changing solution ionic strength. Thus, a lower concentration of PEG can be used to achieve higher precipitation yield of the protein at a lower ionic strength of the solution as compared with that obtained by use of a higher concentration of PEG at a higher solution ionic strength. This is beneficial as it leads to an overall decrease in the amount of PEG required for the precipitation of proteins, hence, lowering the amount of residual PEG in the precipitate.

Figure 2 shows the effect of pH on the solubility of IFNα2a in the presence of 4% wt/vol PEG 1450. As seen, a broad minimum in the solubility of IFNα2a solubility is observed in the pH range of 6.0 to 8.0 in presence of PEG 1450. This shows that the minimum solubility of the protein in presence of PEG is observed in the region of pI of the protein (6.0-7.0). Hence, the solubility of IFNα2a in the presence of PEG is not significantly altered in the range of ~2 pH units around the pI of the protein (pH 6.0-8.0) providing flexibility in order to select a pH that suits best to the protein formulation from stability point of view.

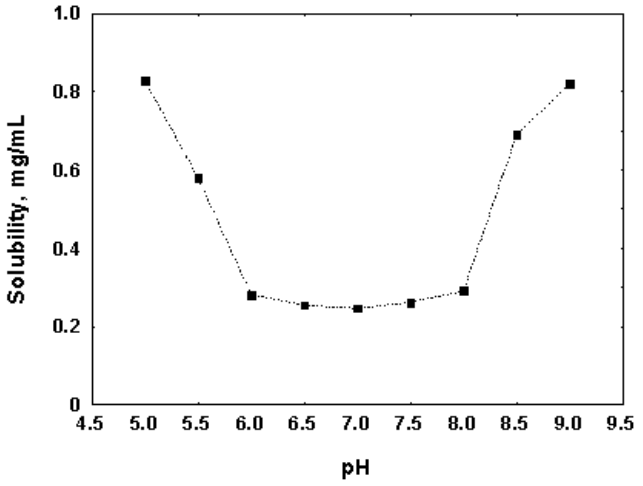

Figure 2. Effect of solution pH on the apparent solubility of IFNα2a in the presence of 4% wt/vol PEG 1450 at 25°C. The data points represent mean of the duplicate studies and the lines are guide to the eyes. The difference between the 2 values in duplicate studies was less than 5% of the mean.

Figure 3 shows the effect of temperature on the solubility of IFNα2a at 5°C, 25°C, and 37°C in presence of PEG 1450 at pH 6.5 and 71-mM solution ionic strength. As seen, temperature has minimal effect on the solubility of IFNα2a in the presence of PEG (4% wt/vol-10% wt/vol) in the given temperature range. This is advantageous since slight fluctuations in the temperature during bulk processing will not affect the precipitation yield of IFNα2a.

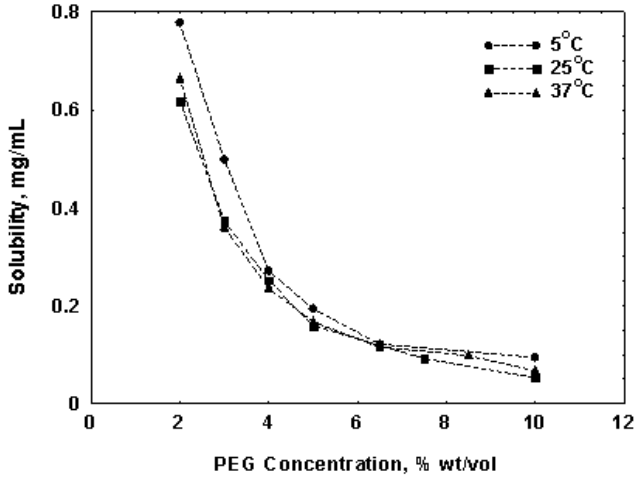

Figure 3. Effect of temperature on the apparent solubility of IFNα2a in presence of varying concentrations of PEG 1450 at pH 6.5 and at a solution ionic strength of 71 mM. The data points represent mean of the duplicate studies and the lines are guide to the eyes. The difference between the 2 values in duplicate studies was less than 5% of the mean.

Overall, it can be concluded from solubility studies that a wide range of PEGs and concentrations can be used to significantly reduce solubility of IFNα2a. So the question arises as to which PEG molecular weight and concentration shall be selected. This depends on several factors. A higher molecular weight PEG is not preferable since these PEGs produce solutions of high viscosity that may become difficult to handle. Based on this, the use of PEG 3450 and other high molecular weight PEGs was disregarded for further studies. Lower molecular weight PEGs (PEG 1000 and lower) were not considered suitable since a higher concentration of these is required to a get similar precipitation yield. Also, these PEGs are usually liquids at ambient temperatures, which could affect the appearance and handling of the final dried product. Hence, based on the above solubility studies, 10% wt/vol PEG 1450 was selected as the suitable precipitant at 71-mM solution ionic strength. Under these conditions, IFNα2a has a very low solubility (0.04 mg/mL) and this solubility is comparable with that obtained by other higher molecular weight PEGs. Use of 10% wt/vol PEG 1450 resulted in a 1:1 wt/wt protein:PEG ratio in the wet precipitate obtained after centrifugation that contained 10 mg of the initial total protein (based on gravimetric analysis).

Structural Characterization of IFNα2a Precipitate

The secondary structure of IFNα2a in the precipitate was compared with that of the native IFNα2a solution using area-normalized second derivative FTIR spectra in the amide I region obtained by ATR-FTIR spectroscopy. As seen in Figure 4, native IFNα2a shows an intense peak at 1654 cm^{-1}, which is a characteristic of the presence of alpha helices in this protein.[44] Upon precipitation of IFNα2a by using 10% PEG 1450, an increase in the intensity of this peak was observed indicating slight changes in the secondary structure of the protein in the precipitated state. The nature of these changes remains uncertain at this point.

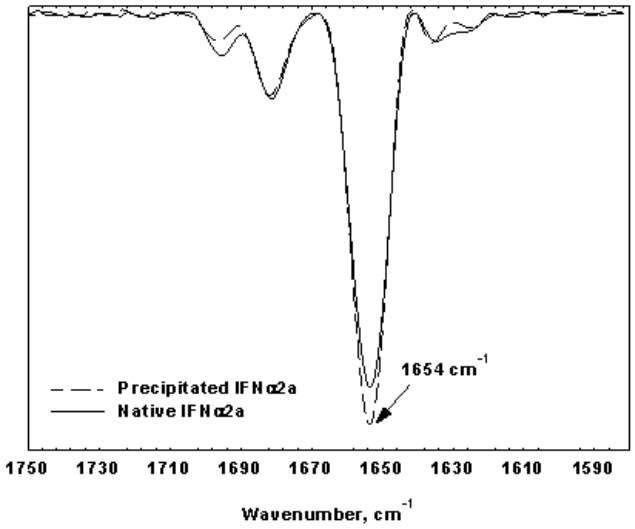

Figure 4. Area-normalized second derivative ATR-FTIR spectra obtained from solution of IFNα2a and of IFNα2a precipitated using 10% wt/vol PEG 1450 at pH 6.5.

In order to ascertain, whether this change in secondary structure was irreversible or reversible, the wet precipitate was redissolved in acetate buffer pH 5.0, and the near UV and far UV CD spectra of the reconstituted sample were compared with that of the original IFNα2a solution. Figure 5 shows these spectra for native IFNα2a solution and reconstituted IFNα2a solution when precipitated from 10% wt/vol PEG 1450 and PEG 8000. The far UV CD spectra indicated that the secondary structure of IFNα2a was retained upon reconstitution, when precipitated from 10% PEG 1450, whereas it was altered when precipitated using 10% wt/vol PEG 8000. The tertiary structure was also altered significantly when precipitated using PEG 8000, whereas only slight changes were observed when precipitated using PEG 1450. These studies indicated that although there were changes observed in the secondary structure of the IFNα2a in the precipitated form as observed from ATR-FTIR studies, these changes were reversible and no significant change in the secondary and tertiary structure was observed in the reconstituted samples when precipitated using 10% wt/vol PEG 1450. These studies also ascertained that it is beneficial to use lower molecular weight PEGs as compared with high molecular weight PEGs, since high molecular weight PEGs can significantly alter the tertiary and secondary structure of proteins. Hence, it was considered safe to use 10% wt/vol PEG 1450 for the precipitation of IFNα2a for all further studies.

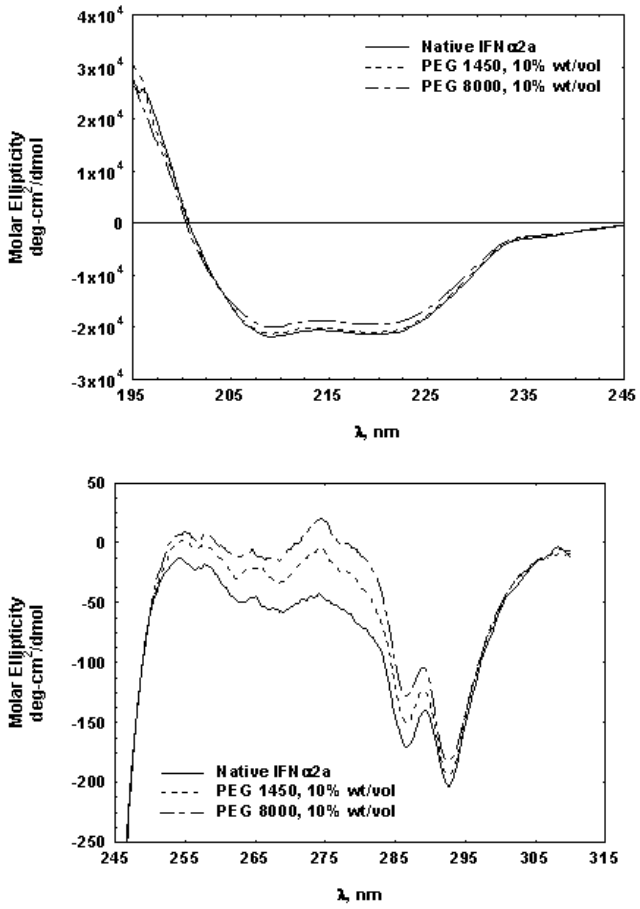

Figure 5. Far UV CD spectra (top) and near UV CD spectra (bottom) of reconstituted IFNα2a in acetate buffer pH 5.0 following precipitation from PEGs.

Vacuum Drying of IFNα2a

The precipitate obtained after centrifugation and removal of supernatant was subjected to vacuum drying in a conventional freeze dryer at a shelf temperature of 25°C and 100 mTorr of pressure for 48 hours. Drying was performed on the wet precipitate containing only

precipitated protein, and on wet precipitate containing IFNα2a mixed with mannitol and/or trehalose in various ratios. Samples were withdrawn at various time intervals during drying and analyzed for residual moisture content and percentage soluble protein after reconstitution of the dried samples.

The temperature profile of the PEG-precipitated IFNα2a inside the vial as compared with the shelf temperature during initial period of drying was monitored using thermocouples. A rapid decrease in the temperature of the product was observed within the first few minutes, which was attributed to the evaporative cooling effect. The temperature then increased due to the net result of heating by the shelf against slow evaporation of water from the sample, until the product reached a temperature similar to that of the shelf temperature, which happened approximately 2 hours into the drying time.

Figure 6A shows the residual moisture content and Figure 6B shows the soluble protein remaining in the samples as a function of drying time. Mannitol and trehalose were added in a 1:100 wt/wt IFNα2a:sugar ratio in these formulations. As observed in Figure 6A, the loss in moisture content is most rapid in protein precipitates containing mannitol. A relatively high amount of moisture (~5% wt/wt) is retained with samples containing only IFNα2a precipitate without any added sugar or in samples containing trehalose. The moisture content observed in trehalose containing samples will have contributions from the undissolved trehalose, which is present as dihydrate in its crystalline form, and from moisture that is retained by the precipitate. In presence of mannitol, most of the moisture was removed within first 2 hours of drying, and the final moisture content was observed to be less than 1% wt/wt. Figure 6B shows that the insoluble aggregates of IFNα2a were formed as drying proceeded in the absence of the sugars. Only 50% of the total protein in the precipitate remained soluble upon 24 hours of drying. No further formation of aggregates was observed, and this correlated with the plateau achieved in the residual moisture content of these samples. This indicated that the formation of aggregates is related to the loss in moisture from the samples upon drying. As most of the moisture is lost from the samples, further aggregation is prevented, which could be attributed to the low mobility in the system. Both mannitol and trehalose were effective in preventing formation of insoluble aggregates at the end of drying.

Table 1 shows the effect of protein:mannitol/trehalose wt/wt ratio on the percentage soluble protein remaining upon reconstitution following end of drying. It was observed that mannitol exhibited its full protective effect at 1:10 or higher IFNα2a:mannitol ratios, whereas in case of trehalose, even 1:2 protein:sugar ratio was sufficient to prevent formation of insoluble aggregates during drying. Although protection of the protein was achieved at lower protein:sugar ratios (1:10 wt/wt IFNα2a:sugar), a higher ratio (1:100 wt/wt IFNα2a:sugar) was selected for the formulation, as this ratio resulted in a powder form of the final product and hence provided better handling of the dried product.

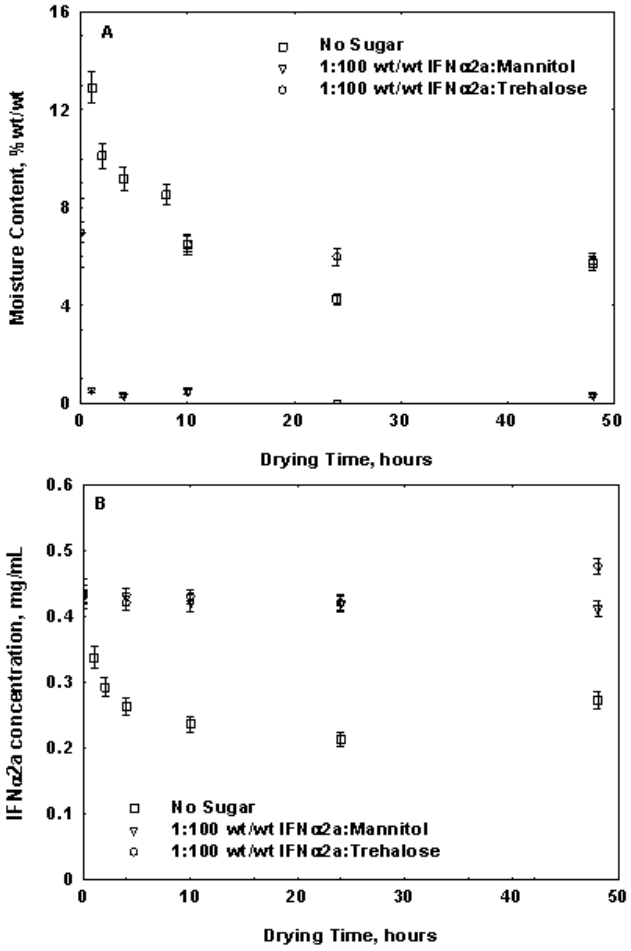

Figure 6. Residual moisture content (A) in the dried samples and soluble IFNα2a remaining (B) in the reconstituted samples at various time points during drying. Drying was performed at a shelf temperature of 25°C and at a pressure of 100 mTorrs for 48 hours.

It is important to note that the final desired wt/wt protein:sugar ratio to obtain a powder form of the product depends strongly on the initial amount of the wet precipitate (ie, the amount of protein precipitated) and the amount of water present in the wet precipitate. For a lower amount of protein, as in the present case, a higher wt/wt protein:sugar ratio (1:100) was required because the wet precipitate contained a lot of water, which dissolved a considerable amount of sugar. In fact, if a

Table 1. Effect of IFNα2a:Mannitol/Trehalose Wt/Wt Ratio on the Soluble Protein Remaining in the Solutions Reconstituted From Samples Vacuum Dried at 100 mTorr for 24 Hours at 25°C

IFNα2a:Mannitol Ratio, wt/wt	Soluble Protein, mg/mL Mean ± SD, n = 3*	Moisture Content, % wt/wt Mean ± SD, n = 3†
Before drying	0.420 ± 0.041 (100%)	-
1:0	0.289 ± 0.024 (68.8%)	5.5 ± 0.64
1:2	0.331 ± 0.036 (78.8%)	-
1:5	0.384 ± 0.033 (91.4%)	-
1:10	0.415 ± 0.039 (98.8%)	1.21 ± 0.21
1:100	0.410 ± 0.027 (97.6%)	1.44 ± 0.33
IFNα2a:trehalose ratio, wt/wt		-
1:0	0.289 ± 0.014 (68.8%)	5.5 ± 0.64
1:2	0.389 ± 0.067 (91.6%)	-
1:100	0.412 ± 0.044 (97.7%)	6.05 ± 0.55

*Percentage recovery as compared with initial prior to drying.

†(-) indicates not determined.

higher amount of protein is precipitated, such as 10 mg or higher, the protein to water ratio increases tremendously and a lower protein:sugar ratio is required to achieve a dry form of the product. For example, if 50 mg of protein is precipitated and collected (studies done using beta-lactoglobulin), a 1:20 protein:mannitol ratio was sufficient to provide a powder-like physical appearance to the final product. This is important since the actual protein:sugar ratio required for stabilization is only ~1:5 wt/wt, and whatever excess is added is only for the purpose of bulk handling of the final product.

Figure 7A and 7B show the effect of mannitol and trehalose, respectively, on the secondary structure of IFNα2a in the dried state, when added in various ratios, as studied by area-normalized second-derivative transmission FTIR spectroscopy. In the absence of sugars, a loss in the intensity of peak at 1654 cm^{-1} was observed, indicating a loss in the alpha-helical content of the protein upon drying. This loss was accompanied by an increase in the intensity of peaks in the 1680 to 1690 cm^{-1} region, which indicated presence of β turns and/or antiparallel β sheets.[45] This finding is attributed to the formation of protein aggregates in the dried samples in the absence of sugars as discussed earlier.

In the presence of mannitol, the intensity of the peak in the 1650 to 1660 cm^{-1} region increased with a shift observed in the peak minima of ~2 cm^{-1}. This shift is usually related to the dehydration of the sample. The extent of increase in the peak intensity varied with the IFNα2a:mannitol ratio; however, in all cases the intensities were found to be closer to peak intensity obtained for the wet precipitated protein as compared with that of the native IFNα2a solution. This finding indicated that IFNα2a retained the secondary structure in the presence of mannitol similar to that of the wet precipitated protein.

In the presence of trehalose, the intensity of the peak observed at 1656 cm^{-1} was independent of the IFNα2a:trehalose ratio and was found to be similar to that of the wet precipitated IFNα2a. This finding indicated that trehalose has a better protective action to preserve the secondary structure of IFNα2a that is independent of the amount of trehalose present in the dried sample.

Trehalose is known to preserve protein structure against dehydration stress due to its high T_g (glass transition temperature) and its ability to act as a water substituent by hydrogen bonding with the protein.[46-48] In the present studies, when IFNα2a was dried with trehalose only in a 1:10 wt/wt ratio, the dried sample exhibited a T_g of 54°C (data not shown). This finding indicated the presence of amorphous trehalose in the dried sample. However, the mechanism of the stabilizing effect of mannitol to prevent protein aggregation during drying was not very clear. In a separate study, where protein-mannitol interactions were studied in greater detail using beta-lactoglobulin as the model protein, it was observed that mannitol crystallization was considerably inhibited in the presence of proteins during vacuum drying. Similar results showing the inhibition of mannitol crystallization

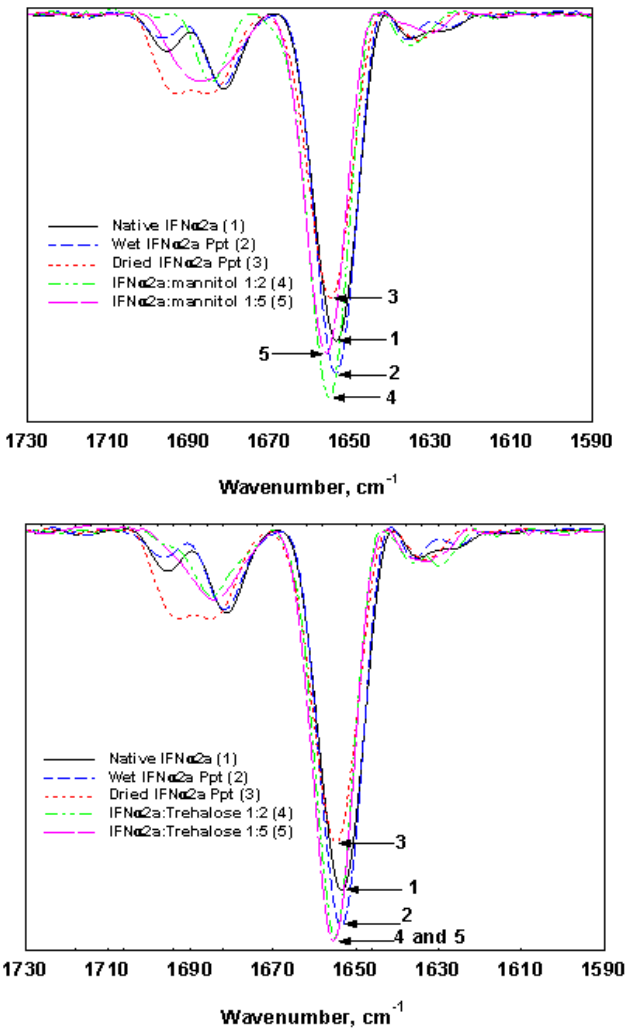

Figure 7. Area-normalized second-derivative FTIR spectra of IFNα2a in solution, wet precipitate of IFNα2a, and vacuum-dried precipitate of IFNα2a in presence of mannitol (top) and Trehalose (bottom). (1) Native IFNα2a solution, (2) Wet IFNα2a precipitate, (3) Dried IFNα2a precipitate, (4) Dried IFNα2a:sugar 1:2 wt/wt, and (5) Dried IFNα2a:sugar 1:5 wt/wt.

by proteins have also been reported during freeze drying[49] or spray drying.[50] These studies provided an indication of how mannitol could have stabilized IFNα2a against aggregation during vacuum drying of the PEG-precipitated protein. When excess of mannitol is added initially to the wet precipitate before drying, some of the mannitol dissolves in the available water surrounding the protein precipitate. During drying, this dissolved mannitol could have interacted with the protein molecules and upon loss of water remained in the amorphous state along with the protein. Thus, the amorphous mannitol formed during the initial stages of drying could have prevented protein-protein interactions from taking place by surrounding protein molecules and thus prevented aggregation upon drying of formulations containing precipitated protein and mannitol only.

It was observed from separate studies that when dried at 25°C for up to 24 hours, amorphous mannitol could not be detected by differential scanning calorimetry (DSC). However, when dried at 5°C, crystallization endotherm of mannitol appeared in the DSC thermograms (data not shown) indicating presence of amorphous mannitol in the initial sample. This finding indicated that the amorphous mannitol that could have formed during initial stages of drying at 25°C, crystallized during prolonged drying up to 24 hours. This was beneficial because the conversion of amorphous mannitol to crystalline mannitol is not desirable upon storage as this may affect the long-term stability of dried protein and, hence, should rather happen during drying. Even though mannitol crystallized during drying, aggregation was still not observed in samples containing mannitol only. This finding was attributed to the rapid loss of water and low residual moisture (~1% wt/wt) in these samples, which provided kinetic stability for the duration of drying.

To check the content uniformity of protein when mixed in a 1:100 wt/wt IFNα2a:mannitol ratio, IFNα2a was precipitated from a solution containing 8 mg of the protein in the precipitate. The precipitate was then collected following centrifugation and then mixed with 800 mg of mannitol. The mixture was vacuum dried at 25°C, 100 mTorr for 24 hours and then divided into 8 equal parts by weight. Each part was then dissolved in 25-mM acetate buffer, pH 5.0, and the protein amount was determined by measuring A_{280} of the solution. The mean IFNα2a content in the dried samples of n = 8 was observed to be 0.844 mg with a standard deviation of 0.024 indicating satisfactory content uniformity.

From the drying studies, it can be concluded that addition of sugars, either mannitol or trehalose, was essential to prevent protein aggregation during vacuum drying. Based on the above results, both trehalose and mannitol were included in the final formulation in the wt/wt ratio of 1:10:100 IFNα2a:trehalose:mannitol. It is speculated that trehalose will provide long-term stability of dried IFNα2a, whereas mannitol will provide better appearance, low moisture content (~1% wt/wt), and better handling of the final product.

Accelerated Storage Stability Studies

For stability studies, IFNα2a was precipitated using 10% wt/vol PEG 1450 and the precipitate was collected after centrifugation and removal of the supernatant. Trehalose and mannitol were added dry to the wet precipitate in

1:10:100 wt/wt IFNα2a:trehalose:mannitol and mixed well. The mixture was divided equally in glass vials, each containing equivalent to 1 mg of IFNα2a, vacuum dried at 25°C, 100 mTorr for 24 hours and finally sealed under vacuum. For comparison, formulations were also prepared containing either 1:100 wt/wt IFNα2a:mannitol or 1:100 wt/wt IFNα2a:trehalose by vacuum drying in a similar way as described above. A lyophilized formulation containing 1:1:10:100 wt/wt IFNα2a:PEG1450: trehalose:mannitol was also kept under various storage conditions to compare the storage stability of the product prepared from vacuum drying as compared with that prepared from lyophilization. Accelerated storage stability studies were performed at temperatures of 40°C and the control samples were stored at –20°C. The stability of IFNα2a was monitored by following formation of insoluble and soluble aggregates and for the formation of the oxidized species of IFNα2a.

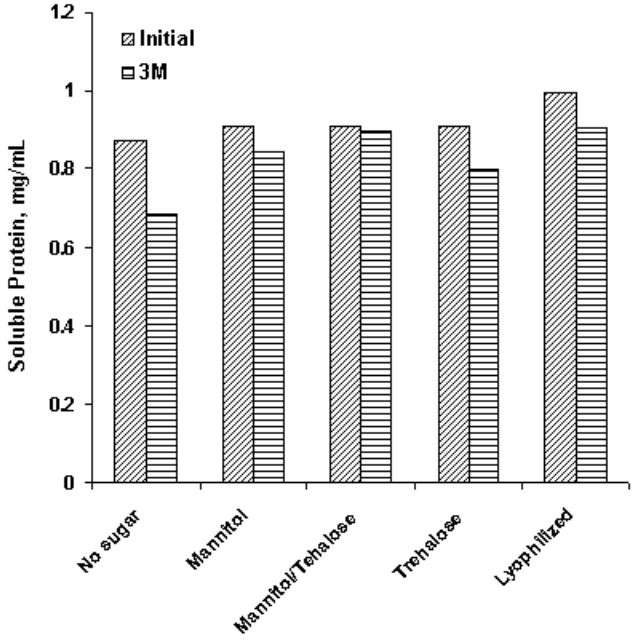

Figure 8. Storage stability of various formulations of PEG-precipitated and vacuum-dried IFNα2a and lyophilized IFNα2a at 40°C indicating soluble protein remaining in the initial samples and in samples after 3 months storage. The histograms represent the mean of duplicate studies. The difference was less than 5% in the duplicate studies.

Figure 8 represents the stability of various samples stored at 40°C (3 months) as indicated by the concentration of the soluble protein remaining in the reconstituted samples. It is clear that the vacuum-dried formulation containing both trehalose and mannitol exhibited maximum stability upon storage at 40°C as compared with other vacuum-dried and lyophilized formulations. This is based on the observation that minimum loss of the protein in the form of insoluble aggregates occurred in this sample as compared with the initial sample. The stability was significantly better than the no-additive formulation and marginally better than other formulations. Hence, this formulation can exhibit a 2-year real-time stability at room temperatures as per ICH stability guidelines.

The protective role of trehalose is well established for the long-term stabilization of proteins in the dried state; however, it failed in the case of vacuum-dried IFNα2a as observed in the present studies. This finding was attributed to the higher moisture content (~5% wt/wt) in these formulations as compared with the formulations that contained mannitol (moisture content was around 1% wt/wt in mannitol-containing dried samples). Similarly, vacuum-dried formulations that contained only mannitol also failed to exhibit desirable stability, and this was attributed to the inability of the crystalline mannitol to provide any protective action against aggregation.

Figure 9 shows the SEC results for the vacuum-dried samples containing both trehalose and mannitol as compared with that of the native IFNα2a at 40°C (3 months). These chromatograms indicated that there was no formation of any soluble aggregates during storage under these conditions. In general, it was observed that even in samples where insoluble aggregates were formed upon storage, the filtered solution did not show presence of any soluble aggregates.

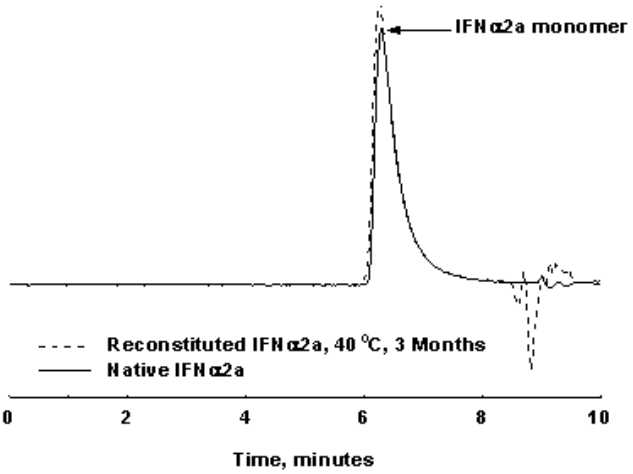

Figure 9. SEC-HPLC chromatogram of native IFNα2a solution and IFNα2a solution reconstituted and filtered from vacuum-dried sample stored at 40°C for 3 months.

Under neutral and acidic conditions, the main chemical pathway of degradation of IFNα2a is the oxidation of the Met residue (Hoffman-La Roche, unpublished data).

Table 2. Percentage Oxidized IFNα2a in Native IFNα2a Solution and in Solutions Reconstituted From Vacuum-Dried and Lyophilized IFNα2a Samples Stored at Various Temperatures. The Results Are Shown for the Formulation Containing IFNα2a:Trehalose:Mannitol in a 1:10:100 Wt/Wt Ratio

Storage Condition	% Oxidized IFNα2a, (SD) n = 3
Initial solution	0.77 (0.023)
IFNα2a solution oxidized by 100-mM H_2O_2 in 5 minutes	34.05 (2.35)
Vacuum dried, 40°C, 2 mo	1.07 (0.06)
Vacuum dried, 40°C, 3 mo	4.16 (0.034)
Lyophilized with 0.1% PEG, 40°C, 3 mo	8.79 (0.54)
Lyophilized, no PEG, 40°C, 3 mo	5.43 (0.43)

Table 2 shows the percentage oxidized IFNα2a in different formulations stored at various conditions. All of the results are shown for the precipitated and dried formulation that contained IFNα2a:PEG1450:trehalose:mannitol in the 1:3:10:100 wt/wt ratio with the total IFNα2a content around 0.8 mg. As shown in Table 2, less than 10% of the oxidized IFNα2a was observed in all of the vacuum-dried formulations. The extent of oxidation was comparable in the vacuum-dried samples and in the lyophilized samples with and without PEG. This was an important observation, since it indicated that the residual PEG that remained in the wet precipitate following centrifugation (~1% wt/wt), and hence was also present in the dried samples, did not induce any significant oxidation of IFNα2a.

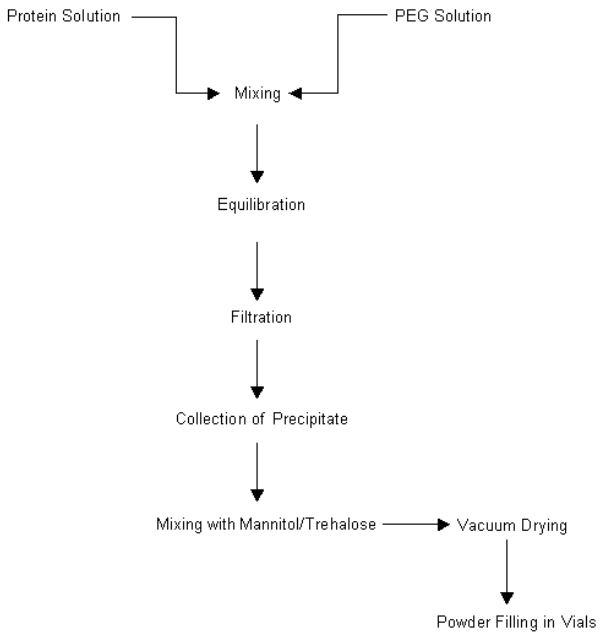

Figure 10. Schematic of the process for formulation of proteins as dry powders based on PEG-induced precipitation followed by vacuum drying.

CONCLUSION

The feasibility of developing a novel process to formulate proteins as dry powders has been systematically investigated and reported in this paper. This process is based on the precipitation of proteins using PEGs, followed by separation of the precipitate and subsequent vacuum drying of the precipitate in presence of sugars as stabilizers. The precipitation of a model protein, IFNα2a, was performed successfully using 10% wt/vol PEG 1450 at 25°C at the isoelectric pH of 6.5 and at a solution ionic strength of 71 mM. The precipitation yield of IFNα2a was affected by PEG molecular weight and concentration as well as by solution pH and ionic strength. Hence, these factors can be manipulated to achieve maximum precipitation yield of any given protein without compromising the protein's native conformation. Vacuum drying of the wet precipitate of IFNα2a alone resulted in the formation of insoluble aggregates. Addition of mannitol or trehalose to the wet precipitate, prior to drying, prevented formation of the insoluble aggregates. Both mannitol and trehalose preserved the secondary structure of IFNα2a during vacuum drying. Accelerated storage stability studies indicated that maximum stability is achieved in vacuum-dried IFNα2a formulations that contained both mannitol and trehalose. Mannitol helped in retaining low moisture content in the dried samples, whereas, trehalose was effective in stabilizing the protein against aggregation. The stability of the PEG-precipitated and vacuum-dried IFNα2a formulations was comparable with the stability of similar lyophilized formulations. Hence, it can be concluded that PEG-induced precipitation of proteins followed by vacuum drying in the presence of suitable stabilizers can be used as a suitable method to formulate proteins as stable dry powders. A proposed scheme of this process that can be used commercially, indicating the various steps involved, is represented in Figure 10. At this point, this process would require a sterile powder filling in an in-

dustrial setup, which may have its own technical difficulties. Currently, work is ongoing in our laboratory to modify the process to make it more amenable to be used in a lyophilization type of setting.

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